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<p>(54) Title: COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY WITH THE ALPHA-3 DOMAIN OF A CLASS I MAJOR HISTOCOMPATIBILITY MOLECULE</p>		
<p>(57) Abstract</p> <p>Composition, and method of its use, including a polypeptide having the α_3 domain, but lacking the α_1 and α_2 domains, of a class I major histocompatibility complex heavy chain polypeptide.</p>		

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DESCRIPTIONCompositions And Methods For Immunotherapy With The
Alpha-3 Domain of a Class I Major
Histocompatibility MoleculeBackground of the Invention

This invention relates to the alpha-3 (" α_3 ") domain, dissociated from the alpha-1 (" α_1 ") and alpha-2 (" α_2 ") domains, of a class I major histocompatibility molecule, and its uses.

Therapeutic compositions and methods for nonspecific immunosuppression have broad clinical relevance for the treatment of alloimmune and autoimmune diseases. Numerous methods for generalized nonspecific immunosuppression have been described in the literature, for example, X-irradiation, cytotoxic drugs, cyclosporin A, and corticosteroids. More selective methods for nonspecific immunosuppression, affecting narrower subsets of the immune cell repertoire, have also been reported, for example, anti-CD4 antibodies (targeting CD4-positive T-cells) for autoimmune and alloimmune disease therapy, and anti-IgE antibodies (targeting IgE-positive B-cells) for allergy therapy.

CD8-positive T-cells are effectors of cytotoxicity in certain diseases, such as alloimmune, autoimmune, and viral diseases. Most CD8-positive T-cells require cell surface CD8 to function as a coreceptor alongside the T-cell receptor, for activation of proliferation and triggering of cytotoxicity when stimulated by antigen-presenting cells (referred to hereinafter as "APCs"). For example, antisense RNA-mediated inhibition of CD8 expression in a CD8-positive human T-cell clone prevents antigen-specific T-cell activation of proliferation and triggering of cytotoxicity in this clone (Hambor, 168 J. Exp. Med. 1237, 1988). Sense gene transfer studies further support CD8's requisite coreceptor function on

CD8-positive T-cells (for example, Dembic, 320 Nature 232, 1986; Dembic, 326 Nature 510, 1987; and Gabert, 50 Cell 545, 1987). Such CD8-positive T-cells requiring CD8 coreceptor function are generally referred to as "CD8-
5 dependent T-cells."

There is evidence that CD8 coreceptor function is dependent upon its binding to a class I major histocompatibility complex (referred to hereinafter as "MHC-I") molecule on the APC. Anti-CD8 antibodies can
10 interfere with the activation of CD8-dependent T-cells. Notably, this antibody-mediated interference is a consequence of both the competitive blocking of CD8:MHC-I interaction (for example, Swain, 78 Proc. Natl. Acad. Sci. USA 7101, 1981; Landegren, 155 J. Exp. Med. 1579, 1982;
15 Spits, 134 J. Immunol. 2294, 1985; Schimonkevitz, 135 J. Immunol. 892, 1985; Goldstein, 138 J. Immunol. 2034, 1987; and Moldwin, 139 J. Immunol. 657, 1987), and direct inhibition of the T-cells, presumably through the cross-linking of CD8 on the T-cell surface (for example, Welte,
20 131 J. Immunol. 2356, 1983; Hunig, 159, J. Exp. Med. 551, 1984; Fleischer, 136 J. Immunol. 1625, 1986; van Seventer, 16 Eur. J. Immunol. 1363, 1986; and Geppert, 137 J. Immunol. 3065, 1986).

MHC-I molecules are each composed of an alpha
25 (hereinafter referred to as " α ") heavy chain noncovalently associated with a β_2 -microglobulin (hereinafter referred to as " β_2m ") light chain. The α heavy chain has three extracellular domains (designated α_1 , α_2 , and α_3), and a transmembrane and cytoplasmic domain (for example,
30 Tykocinski, 133 J. of Immunology 2261, 1984). X-ray crystallographic data has established that the polymorphic α_1 and α_2 domains interlock to form a nominal antigen peptide binding structure. The non-polymorphic α_3 domain bridges the interlocked $\alpha_1:\alpha_2$ unit and the membrane, and is
35 structurally homologous to immunoglobulin constant region domains (Bjorkman, 329 Nature 506, 1987). The

noncovalently associated β_2m light chain contacts both the $\alpha_1:\alpha_2$ and α_3 structural units of the MHC-I heavy chain.

In general, it has been thought that β_2m association is important for intracellular transport (Williams, 142 J. Immunol. 2796, 1989; Townsend, 324 Nature 575, 1986; Krangel, 18 Cell 979, 1979), nominal antigen peptide binding (Townsend, 62 Cell 285, 1990; Kozlowski, 349 Nature 74, 1991; Rock, 88 Proc. Natl. Acad. Sci. USA 301, 1991; Boyd, 89 Proc. Natl. Acad. Sci. USA 2242, 1992), and conformational stability (Allen, 83 Proc. Natl. Acad. Sci. USA 7447, 1986; Rock, 88 Proc. Natl. Acad. Sci. USA 4200, 1991; Hansen, 140 J. Immunol. 3522, 1988; Otten, 148 J. Immunol. 3723, 1992; Mage, 89 Proc. Natl. Acad. Sci. USA 10658, 1992).

Williams et al., 11 Immunol. Res. 11, 1992 describe modulation of T-cell responses with peptides derived from the α helical region of MHC class II molecules; that is the antigen-binding groove. The authors state: "Peptides derived from the amino acid sequences of these helices may be capable of modulating immune responses and aiding in the dissection of immune recognition."

Zimmerman and Elliot, WO 89/12458 and Sharma et al., WO 89/12459 describe various conjugates of MHC molecules with peptides.

25 Summary of the Invention

The α_3 domain noncovalently binds β_2m and CD8. Although it has been suggested that the CD8 and β_2m binding sites are on opposite sides of the α_3 domain, it was not previously known whether the binding of CD8 and β_2m to the α_3 domain is interdependent; more specifically, whether β_2m association is required for CD8 association, and whether additional contact sites within the α_1 and/or α_2 domains are required for effective CD8 binding to MHC-I.

Applicant has discovered that the MHC-I α_3 domain can be produced as an independent functional unit, dissociated from the α_1 , α_2 , transmembrane and cytoplasmic domains of

the class I MHC heavy chain, as well as from β_2m . Significantly, the α_3 domain unit folds properly in the absence of its natural flanking sequences within the MHC-I heavy chain and in the absence of β_2m , and in so doing, it
5 retains the capacity to bind to CD8 (the α subunit, and perhaps the β -subunit). Hence, Applicant has discovered that β_2m association is not obligatorily required for CD8 association with the MHC-I α_3 domain. The α_3 domain minimal unit structure provides a suitable reagent for
10 binding CD8 molecules and CD8-positive cells in therapeutic and diagnostic contexts.

The invention features polypeptide compositions which share in common the presence of a complete MHC-I α_3 domain, dissociated from the normally contiguous α_1 and α_2 domains
15 able to bind CD8. The compositions included in the invention include both soluble and membrane-binding α_3 -containing polypeptide (referred to hereinafter as " α_3 polypeptides") variants formed by standard procedures, as described below.

20 Soluble α_3 polypeptides consist of either the MHC-I α_3 domain alone (monomeric or multimeric), or this domain covalently linked (for example, in a chimeric polypeptide) to a second molecular unit which confers to the chimeric molecule desired properties. For example, the second
25 molecule can be any one of a number of antigenic polypeptides that are well known to those in the art, including the maltose binding protein and viral hemagglutinin sequences. Such antigenic polypeptides simplify purification of the α_3 polypeptide by affinity
30 chromatography. If they include a protease cleavage site, they can be readily proteolytically removed from the functional α_3 domain unit following purification. Another example of a second molecule is a toxin; such α_3 polypeptide:toxin chimeras can be used effectively for
35 eliminating CD8-positive cells from cellular populations. Other antigenic polypeptides include those which stabilize the α_3 -domain, e.g., an Fc region which prolongs the

half-life of the α_3 domain in serum. If such a polypeptide contains at least three histidines then it can be purified on a nickel-SEPHAROSE column.

Membrane-binding α_3 polypeptides are chimeras consisting of the MHC-I α_3 domain linked to a membrane anchor, for example a glycosyl-phosphatidylinositol (hereinafter referred to as "GPI") anchor, or a non-MHC or MHC transmembrane domain.

The invention also includes expression vectors for producing such α_3 polypeptides or chimeras. Though it is known that many eukaryotic polypeptides will not fold properly in prokaryotes, the MHC-I α_3 domain does. Hence, α_3 polypeptides can be produced in both prokaryotes and eukaryotes. This constitutes a significant advantage for α_3 polypeptides over alternative CD8-binding molecules, such as antibodies, in that the former can be produced in a cost-effective fashion via prokaryotic expression systems.

The invention features therapeutic methods that employ α_3 polypeptides. Some of these methods take advantage of the capacity of the α_3 polypeptide or chimeras to bind to CD8 at cellular surfaces and effectively compete for the functional interaction between this cell surface-associated CD8 on T-cells and MHC-I on APCs. When administered in vivo to a patient suffering from CD8-positive T-cell-mediated cytotoxicity, the α_3 polypeptide will competitively inhibit the activation and triggering of cytotoxicity in CD8-positive T-cells. Thus, this method provides an effective immunosuppressive therapy directed specifically at the CD8-positive T-cell subset. Since cytotoxic CD8-positive T-cells are pathogenic in a wide range of diseases, including alloimmune, autoimmune, and certain infectious diseases such as human immunodeficiency virus infection, competitive blockade of CD8-positive T-cell activation is of considerable therapeutic benefit. The α_3 polypeptides are particularly beneficial in the context of acute

pathogenic processes, for example, autoimmune disease flares, wherein recombinant α_3 polypeptides can be administered to the patient in repeated doses.

The invention also features the use of α_3 multimers to
5 direct inhibitory signaling of CD8-positive T-cells directly through their surface CD8. Anti-CD8 antibodies are known to mediate inhibitory signaling in CD8-positive T-cells. Multimeric α_3 polypeptides mimic anti-CD8
10 antibodies in the direct nonspecific inhibition of CD8-positive T-cells. The α_3 multimers offer advantages over antibodies in this context, including the absence of antigenic immunoglobulin sequences. The α_3 multimers to be used do not contain any antigenic components.

The invention also features therapeutic methods for
15 treating patients with lymphomas expressing CD8 at their surface. α_3 polypeptides can be used to target toxins, such as ricin, Pseudomonas and Staphylococcal endotoxins to CD8-positive lymphoma cells. α_3 :toxin chimera conjugates can be produced using chimeric coding sequences
20 in prokaryotic expression systems. Such conjugates can be used in several clinical contexts. A preferred clinical application is the in vitro purging of CD8-positive lymphoma cells from bone marrow in the course of autologous bone marrow transplantation. In vivo tumor
25 therapeutic applications involve situations wherein transient deficits in the peripheral CD8-positive T-cell population will not be problematic, for example, patients who are to undergo immunosuppressive therapy prior to autologous bone marrow transplantation.

30 The invention also includes use of α_3 polypeptide:toxin conjugates to eliminate normal CD8-positive T-cells from cellular populations both in vitro and in vivo. A preferred in vitro method is pre-treating allogeneic bone marrow to be transplanted, in order to eliminate
35 alloreactive CD8-positive T-cells that can contribute to graft-versus-host disease. This therapy can be combined with anti-CD4 antibody therapy that is currently used to

eliminate CD4-positive T-cells from immune cell populations.

The invention also concerns diagnostic methods for detecting CD8 polypeptides. The method utilizes α_3 polypeptides as detecting reagents. A major advantage of α_3 polypeptides over the more conventional antibodies used in this context is the economy of producing large amounts of α_3 polypeptide via prokaryotic expression systems. Soluble CD8 is known to be elevated in the course of a variety of infectious and other diseases. α_3 polypeptides can be incorporated into ELISA or other conventional assays for the detection of soluble CD8 in serum samples from patients suffering from diseases with this diagnostic marker. α_3 polypeptides can also be used in cellular diagnostic assays to detect CD8-positive cells. Biotinylated or fluoresceinated α_3 polypeptide derivatives are suitable for such detecting reagents. In addition, α_3 polypeptides can be tagged in conventional ways to permit their use for the in vivo detection of solid tumor masses containing CD8-positive tumor cells.

The invention also allows production of anti- α_3 domain-specific antibodies using the isolated α_3 domain unit as an immunogen. This provides an effective means for producing such antibodies and circumvents the generation of α_1 and α_2 domain-specific antibodies when the complete extracellular domain of MHC-I is used as an immunogen.

In other aspects, this invention features methods for purification of polypeptides comprising CD8 from mixtures of proteins. α_3 can be used as a ligand on solid-phase supports for affinity purification procedures.

Thus, in a first aspect, the invention features a method for treating a patient suffering from an autoimmune, alloimmune, or viral disease with aberrant CD8-positive T-cell cytotoxicity, by administering to the patient a reagent comprising an MHC-I α_3 domain (and not

the α_1 or α_2 domains), to thereby block activation of the cell.

In preferred embodiments, the reagent comprises, consists of, or consists essentially of, a compound
5 selected from an isolated α_3 domain; an α_3 domain linked to an antigenic polypeptide; an α_3 domain linked to biotin or fluorescein; an α_3 domain linked to a toxin; an α_3 domain linked to a GPI or Fc moiety; and an α_3 domain linked to a hydrophobic polypeptide transmembrane extension. The
10 reagent preferably competitively inhibits binding of natural MHC-I to CD8 α or β .

By "consisting essentially of" is meant to include those peptides or chimeras in which the CD8 binding portion of α_3 is provided free from the other MHC α
15 regions, although short regions of about 5 - 10 amino acids may be present so long as they do not affect the CD8 binding ability of the peptide.

In a second aspect, the invention features a method for treating a patient with a CD8-positive T-cell
20 lymphoma, by eradicating tumor cells with an α_3 :toxin conjugate, either in vitro or in vivo. The method includes contacting the conjugate with the target cells to be killed under suitable killing conditions well known to those in the art.

25 In a preferred embodiment, the method is a method for purging CD8-positive lymphoma cells from a bone marrow specimen to be used for autologous bone marrow transplantation; other methods include the use of an α_3 :toxin conjugate in vivo to eradicate tumor cells.

30 In a third aspect, the invention features diagnostic methods for detecting CD8 in vitro or in vivo by detecting binding of the α_3 peptide to CD8 α cells or soluble CD8. Such a method is useful for detection of the presence and amount of CD8 conjugates used in therapy. See Hambor et
35 al., supra.

In one example, the α_3 polypeptide is incorporated into an ELISA assay to detect soluble CD8 in patients'

sera. In another example, the α_3 polypeptide is conjugated to a reporter molecule such as biotin, fluorescein, or radionuclide and used to detect CD8-positive normal or tumor cells in vitro or in vivo.

5 In a fourth aspect, the invention features a method for producing α_3 polypeptides using an expression vector comprising sequence encoding a MHC-I α_3 domain.

In preferred embodiments, the vector is selected from a prokaryotic expression vector and a eukaryotic
10 expression vector.

In a fifth aspect, the invention features a method for producing MHC-I α_3 domain-specific polyclonal and monoclonal antibodies by use of the α_3 domain only as an immunogen.

15 In a sixth aspect, the invention features a method for purifying polypeptides comprising CD8 sequences, by using the α_3 domain bound to a solid support for affinity purification.

Other features and advantages of the invention will
20 be apparent from the following description of the preferred embodiments of the invention, and from the claims.

Description of the Preferred Embodiments

The following are examples of production of the α_3
25 domain isolated from MHC class I sequences. These examples are not limiting in the invention. Those in the art will recognize many equivalent methods and compositions within the pending claims.

Example 1: Production of recombinant α_3 :maltose-binding 30 protein chimeric polypeptide

The α_3 domain of human MHC-I was produced as a fusion protein, linked to the maltose-binding protein (referred to hereinafter as "MalE"), in order to simplify purification of the α_3 polypeptide. MalE fusion proteins
35 can be readily purified on amylose columns. To optimize

production of properly-folded protein product, away from the reducing microenvironment of the bacterial cell cytoplasm, the α_3 :MalE polypeptide was expressed with a prokaryotic vector system which targets the polypeptides of interest to the outer cytoplasmic wall and the less reducing microenvironment of the periplasmic space. The specific example here provides an indication of the region referred to herein as α_3 MHC I. See Bjorkman et al., 329 Nature 506, 1987, hereby incorporated by reference herein.

Those in the art will recognize that slight variations (± 5 amino acids) can be readily devised using similar methodology.

M13mp18/A2.1 (from J. Nuchtern, N.I.H. see Koller and Borr, 134 J. Immunol. 2727, 1985), a cDNA encoding full-length HLA-A2.1, was used as a source of a MHC-I α_3 domain. An EcoRI insert containing the entire HLA-A2.1 cDNA was subcloned into pBluescript II KS (Stratagene) to generate pHLA-A2.1/BT. A partial cDNA encoding the α_3 domain of HLA-A2.1 only, was generated by PCR using 5'-TAGGATCCATGGACGCCCCCAAAC-3' and 5'-TAGAATTCTCACCATCTCAGGTGAGG-3' as 5' and 3' primers, respectively, and pHLA-A2.1/BT plasmid as template. A start codon, as well as a BamHI (GGATCC) restriction endonuclease site, were incorporated into the 5'-end of the 5' primer, and a stop codon and another restriction endonuclease site (EcoRI) were incorporated into the 5'-end of the 3' primer. PCR reactions and thermocycling were carried out using standard procedures (see example 2 below). Following organic extraction, the digested α_3 cDNA insert was subcloned between the BamHI and EcoRI sites in the multiple cloning site of the baculovirus vector pVL1393 (Invitrogen, San Diego, CA), re-mobilized with BamHI/BglII digestion, and subcloned into the prokaryotic expression vector pMALp (New England Biolabs, Beverly, MA) which carries the MalE coding sequence adjacent to the multiple cloning site of the vector. This generated a chimeric coding sequence in which α_3 and maltose binding protein are linked in-frame.

The resulting α_3 :MalE expression vector was designated pA2.1 α_3 :Malp.

For large-scale purification, α_3 -MalE was purified from 2-liter cultures of E. coli DH5 α (ATCC strain) transformants. Bacterial transformants were grown in LB containing 0.2% glucose and 100 mg/ml ampicillin up to O.D.₆₀₀ = 0.4 and induced with IPTG (GIBCO; 0.3 mM final concentration) to maximize fusion protein production. Following induction, bacteria were pelleted, washed with phosphate buffered saline (PBS), and 6.5 grams of bacteria (wet weight) were resuspended in 50 ml lysis buffer (10 mM phosphate/30 mM NaCl/0.25% Tween 20/10 mM 2-ME/10 mM EDTA/10 mM EGTA) supplemented with 50 mg lysozyme (Sigma). After 30 minutes on ice, samples were divided in half, and each half was sonicated 3" to shear high molecular weight DNA. Lysates were clarified by centrifugation (9000g X 30"; followed by 14000g X 30"), and supernatants were harvested and diluted 1:5 in column buffer (0.25% TWEEN 20/10 mM phosphate/0.5 M NaCl/1mM NaN₃/1 mM EGTA). Fusion protein was then bound to a 75 ml amylose resin column (NE Biolabs), the column was washed with three column volumes of column buffer and five column volumes of column buffer without detergent. Fusion protein was eluted with column buffer (without detergent) containing 10 mM maltose. Fractions were analyzed by spectrophotometry and by SDS-PAGE analysis. Fusion protein-containing fractions were dialyzed overnight against 20 mM Tris-Cl/0.1 M NaCl/2 mM CaCl₂/1mM NaN₃ and concentrated with Centriprep 30 microconcentrators (Amicon).

Electrophoretic analysis by 12% SDS-PAGE and Coomassie Blue staining documented the production of an intact fusion protein of approximately 46 kD, as expected, which contrasted with the molecular weight of nonfused MalE produced by the vector (approximately 30 kD).

The identity of the α_3 :MalE fusion protein was confirmed by additional methods. When bound to plastic, both the fusion protein and the native nonfused peptide

Male could be detected with a rabbit antiserum directed against Male. In western blot analysis, a biotinylated form of monoclonal antibody m100038 (anti-HLA I ABC HC) (Olympus), which has specificity for the α_3 domain MHC-I, could bind the fusion protein product, but not negative control proteins. The α_3 domain can also be readily expressed in other systems, including the Bacullovirus and yeast expression systems.

Example 2: Production of recombinant sCD8 α

For purposes of sCD8 α production, we chose the eukaryotic glutamine synthetase amplification/expression (GS) system due to the rapidity with which amplified transfectants can be derived. This expression system has been used for purifying other cell surface proteins, including soluble CD4 derivatives.

pT8F1 includes a cDNA encoding full-length human CD8 α (see, Litman, 40 Cell 237, 1985). A truncated cDNA encoding the signal peptide and amino-terminal 150 amino acids of CD8 α , was generated by PCR using CD8 α -specific 5' and 3' primers, and pT8F1 plasmid as template. Three restriction endonuclease sites (BamHI, HindIII and XhoI) were incorporated into the 5'-end of the 5' primer, and a stop codon and another three restriction sites (SalI, XhoI and HindIII) added to the 5'-end of the 3' primer. PCR reactions were performed under mineral oil in the presence of 10 mM Tris-HCl pH 8.3/ 50 mM KCl/1.5 mM MgCl₂/0.01% gelatin/1.25 mM of each deoxynucleotide triphosphate/0.1 nmoles of each primer/2.5U AMPLITAQ DNA polymerase (U. S. Biochemical, Cleveland, OH). Reaction thermocycling (30 cycles at 94°C for 1", stepped to 50°C for 2", stepped to 72°C for 2") was performed in a PTC-100 thermocycler (M.J. Research, MA). Following organic extraction, the amplified CD8 segment was subcloned into the prokaryotic vector BLUESCRIPT (Stratagene), the sequence was confirmed, the insert was mobilized again with restriction endonuclease digestion and subcloned into the appropriate

sites of the glutamine synthetase amplification/expression vector pEE14 (CellTech, Ltd.) to generate pCD8 α (sol)/EE14.

pCD8 α (sol)/EE14 was transfected into CHO-K1 cells (ATCC) using lipofectin reagent (Gibco-BRL). Cells were
5 initially selected in the presence of the glutamine synthetase inhibitor methoxy-sulfoximine (MSX; 25mM) (Sigma). Medium for the CHO-K1 cells was DMEM (Whittaker) without glutamine and supplemented with 4.5 gm glucose, 10% FCS (Sigma), pyruvate, nonessential amino
10 acids, glutamate and asparagine, and penicillin/streptomycin. Resistant colonies were cloned using cloning cylinders and then amplified with graded amounts of MSX. Amplified colonies were screened for production of CD8 α using an ELISA (T Cell Diagnostics).

15 Individual clones were then selected for growth in bulk culture. Cloned and amplified transfectants expressing high amounts of sCD8 α in the initial screening period were used to seed roller bottles. Selected clones were seeded into 850 cm² roller bottles (Falcon) and
20 allowed to condition CHO media (without glutamine) for 5-10 days. Harvested media was filtered through 0.45mm filters and then batch adsorbed with 10 gm of anti-Leu2a immunosorbant beads for 16 hr at 4C. Immunosorbant beads were then extensively washed with DPBS (Whittaker).
25 Washed beads were eluted with 0.2M glycine pH 2.5/10mM CHAPS (Sigma). Eluted fractions of 1 ml each were collected directly into 0.4 ml of 0.2 M dibasic sodium phosphate to neutralize them. Eluted CD8 polypeptide was dialyzed against PBS and concentrated using a Centri-prep
30 10 membrane (Amicon). The purity of the eluted product was assessed by 12% SDS-PAGE analysis, followed by silver staining (Biorad). The average α_3 :MallI yield was 0.4 to 2.0 mg per liter of culture (approximately 10⁸ CHO-K1 cells).

35 Analysis of immunoaffinity-purified sCD8 α by silver staining of a nonreducing SDS-PAGE gel revealed that the protein product consisted of a mixture of both dimeric

(approximately 56 kD) and monomeric (approximately 28 kD) forms. The sCD8 α dimer merged into the faster-migrating monomeric form upon reduction. Results from radioimmunoprecipitation of transfected CHO-K1 cells as well as those of human erythroleukemia K562 cells (ATCC) transfected with the same sCD8 α coding sequence, subcloned in this case into the Epstein-Barr virus episomal expression vector pREP9 (Invitrogen, San Diego, CA) which carries the neo^R gene, were identical. In both transfected cell lines, supernatant and cellular lysates contained a mixture of both monomeric and dimeric sCD8 α . These findings support the view that these transfected cell lines secrete this particular truncated form of sCD8 α in both monomeric and dimeric forms and that they are not simply an artifact of the protein purification system.

In a series of soluble truncation mutants of CD8 α , another group of investigators found that CHO cells transfected with a cDNA encoding sCD8 α truncated at amino acid 146 secreted a dimeric polypeptide product. In contrast, CHO cells transfected with a longer cDNA encoding the full extracellular domain of CD8 α (through amino acid 162) secreted a monomeric product. It is therefore not surprising that our polypeptide product from a cDNA encoding the first 150 aa of CD8 α should consist of a mixture of both monomer and dimer.

Example 3: α_3 :MalI effectively binds ¹²⁵I-sCD8 α

The interaction between plate-bound α_3 :MalE and sCD8 α was characterized. Immulon 4 microtiter wells were coated with varying amounts of α_3 :MalE, blocked with BSA, and incubated with ¹²⁵I-sCD8 α at 37°C. For these assays sCD8 α was radio-iodinated using 0.8 mCi Na-¹²⁵I (Amersham) using a standard lactoperoxidase labeling method. Variable amounts of either α_3 /MBP or a control protein were bound to wells of Immulon 4 strips (Dynatech) for 16h at 4°C. Wells were then washed with de-ionized, distilled water and blocked with 0.5% BSA in DPBS for 2h at 37°C. After

an additional washing step, wells were incubated with ^{125}I -sCD8 α in 0.5% BSA/DPBS in a total volume of 0.1 ml for 5h at 37°C. In some experiments, either nonradioactive "cold" sCD8 α or monoclonal antibody (100 ug/ml) were added. After incubation, wells were aspirated, washed twice with 0.1% Tween 20 in DPBS, separated from other wells on the strip, and counted in a gamma counter.

Increased binding was observed between ^{125}I -CD8 α at concentrations between 0.1 μg and above of plate-bound α_3 :MalI. All subsequent experiments were performed using 10 mg of α_3 :MalI to coat the microtiter wells. Binding between ^{125}I -sCD8 α and plate-bound α_3 :MalI was optimal at 5h; subsequent experiments were terminated after a 5h incubation. Binding was shown to be temperature-sensitive, with essentially no binding during a 5h 4°C incubation.

In order to demonstrate that the interaction between these two polypeptides was saturable, graded amounts of non-radioactive sCD8 α were used to inhibit the binding between ^{125}I -sCD8 α and α_3 :MalI. Approximately 100-fold excess "cold" sCD8 α was necessary to inhibit binding down to background levels. The K_d of interaction between sCD8 α and α_3 :MalI was calculated by Scatchard plot analysis to be 2×10^{-7} . This K_d is an order of magnitude greater than the estimated K_d of 3×10^{-6} for the interaction between CD4 and the β_2 domain of class II MHC.

Example 4: Monoclonal antibodies directed against either sCD8 α or MHC α_3 inhibit binding

In order to verify that the sites on MHC-I α_3 and CD8 which govern binding in the cell-free physical binding assay are identical to those which are involved in cellular interactions, we used a panel of monoclonal antibodies directed against either CD8 or MHC-I to inhibit binding.

The hybridomas anti-Leu 2a and PYAD1 B8 were gifts of Dr. R. Evans (Rosewell Park Memorial Institute) and Dr. S.

Y. Yang (Sloan Kettering Memorial Institute), respectively. The hybridomas OKT8, W6/32, BBM.1 were obtained from the American Tissue Type Culture collection. Medium for the hybridoma cells was DMEM (Whittaker) without glutamine and supplemented with 4.5 gm glucose, 10% FCS (Sigma), pyruvate, nonessential amino acids, glutamate and asparagine, penicillin/streptomycin, and glutamine.

Monoclonal antibodies were purified from mouse ascites using a protein A/Affi-gel Immunoglobulin Purification Kit (Bio-Rad). To prepare CD8 immunosorbant, approximately 50 mg of protein A-purified anti-Leu2a was coupled to 15 gm cyanogen bromide-activated Sepharose 4B CL (Pharmacia) using standard methods.

The three monoclonal antibodies directed against CD8 α (anti-Leu2a, OKT8, and PYAD1 B8) substantially inhibited binding between plate-bound α_3 :MalI and ^{125}I -sCD8 α , although only OKT8 inhibited binding to background levels. Control isotype-matched monoclonal antibody did not inhibit binding. In addition, monoclonal antibody m100038 (anti-HLA HC), which can be used to detect α_3 :MalI on Western blots, also inhibited binding. The concentration of mAb used to inhibit binding in this assay, though high (100 mg/ml), is no different from the concentration necessary to inhibit the binding between plate-bound sCD8 α and intact class I-bearing cells (Alcover, 30 Mol. Immunol. 55, 1993). Monoclonal antibody W6/32, which has been shown to inhibit binding between CD8-positive cells and MHC-I-bearing cells in a number of assay systems, did not inhibit the binding between ^{125}I -sCD8 α and α_3 :MalI. The other anti-MHC-I antibodies, known to interact with the α_1 and α_2 MHC-I domains, also did not block the binding to α_3 :MalI, as expected.

Methods

Applicant has demonstrated specific, saturable binding between an α_3 polypeptide and a soluble form of CD8 α at an appreciable affinity of interaction. The specificity of this interaction was confirmed using monoclonal antibodies directed against either CD8 α or against the α_3 domain. Significantly, monoclonal antibodies directed against other sites on MHC-I and β_2m did not block binding, confirming the conclusion that the α_3 domain is functioning as an independent unit.

The discovery that an isolated MHC-I α_3 domain retains the CD8 binding capacity of intact MHC-I leads to novel therapies for inhibiting pathogenic CD8-positive cytotoxic T-cell responses which employ α_3 polypeptides for modulating CD8 function on said cells. Previously, anti-CD8 antibodies were the only reagents considered for modulating CD8 function on CD8-positive T-cells. α_3 polypeptides, as described in the present invention, offer a number of advantages over anti-CD8 antibodies for purposes of modulating cell surface CD8 function. For example, in contrast to antibodies, α_3 polypeptides are smaller molecules, can be readily produced in quantity using prokaryotic expression systems, are less immunogenic, and directly compete for the precise binding site on CD8 for native MHC-I. Moreover, α_3 polypeptides, as described in the present invention, offer a number of advantages over soluble MHC-I molecules that incorporate the α_1 and α_2 domains along with the α_3 domain for purposes of modulating cell surface CD8 function, for example, in contrast to $\alpha_1:\alpha_2:\alpha_3$ multidomain polypeptides, α_3 (single domain) polypeptides are smaller, are more effective competitive inhibitors, can be readily produced in quantity using prokaryotic expression systems, do not require β_2m for proper transport and folding, and lack the polymorphic α_1 and α_2 domains, thereby avoiding allo-immunogenicity problems in patients.

The finding that the α_3 domain retains CD8 binding capacity even when covalently linked to a second molecule, for example, maltose binding protein, indicates that a wide array of functional α_3 polypeptides can be produced which comprise alternative second molecules. Second molecules can be selected which convey to the α_3 polypeptides desired properties. For therapeutic applications, the second molecule can be a toxin or radionuclide to be delivered to transformed (tumor) or nontransformed, for example, alloreactive, CD8-positive cells. For diagnostic applications, the second molecules can include fluorochromes, biotin (for binding avidin conjugates), radionuclides, or any of a large array of epitope tags or antigenic polypeptides that are well-known to those familiar with the art.

There follow examples of the therapeutic and diagnostic methods of the invention below. These examples are not limiting in the invention and those of ordinary skill in the art will recognize that many equivalent methods and reagents can be discovered within the scope of the claims.

Cytotoxic T-cell Immunosuppressive Therapy - In Vivo

Patients with pathogenic CD8-positive cytotoxic T-cells are candidates for immunosuppressive therapy using α_3 polypeptides. Although there are presently no known diseases caused by other (non-T) CD8-positive cells in the body, for example, subsets of natural killer cells and dendritic cells, patients with such diseases would also be candidates for immunosuppressive therapy using α_3 polypeptides and could be treated with α_3 polypeptides by methods which parallel those provided in the present invention. Pathogenic CD8-positive cytotoxic T-cells occur in a variety of disease conditions, including alloimmune, autoimmune, and infectious diseases. For example, CD8-positive T-cells are also known to contribute significantly to allograft cytodestruction in the course

of sub-acute graft rejection, CD8-positive T-cells are also known to contribute to autoimmune cytodestruction in diseases such as multiple sclerosis and inflammatory bowel disease; CD8-positive T-cells have also been implicated in
5 the extensive cytodestruction of both human immunodeficiency virus-infected and non-infected CD4-positive T-cells that occurs in the course of acquired immunodeficiency diseases. Hence, α_3 polypeptides can be used to abrogate the pathogenic effects of CD8-positive
10 cytotoxic T-cells in these diseases.

The first step is to identify a patient in need of cytotoxic T-cell immunosuppressive therapy. Preferred diagnostic methods for accomplishing this are well known to those familiar with the art, and entail the
15 determination of whether a patient suffers from one of the diseases known to be aggravated by CD8-positive T-cell-mediated cytodestruction.

α_3 polypeptides can be used to competitively inhibit CD8-positive T-cell activation and triggering of
20 cytotoxicity. Such therapy is of particular benefit in those clinical settings where a transient halt to an acute flare-up of a disease process is needed. Pharmaceutical compositions comprising α_3 polypeptides are administered to patients by methods appropriate for polypeptide
25 pharmaceuticals, and such methods are well-known to those familiar with the art. Moreover, strategies for optimizing in vivo dosing schedules for patients to be treated are well-known. The amount to be administered can be determined by routine experimentation and optimization,
30 and is generally between 0.1 and 1000 $\mu\text{g/kg}$ animal/day.

As an alternative to using α_3 polypeptides as competitive inhibitors of CD8-positive T-cell activation, α_3 polypeptides can be used to induce a persistent non-responsive state in those cells. It is known that cross-
35 linking of surface CD8 on T-cells using anti-CD8 antibodies leads to a non-responsive state in the cells. Multimeric α_3 polypeptides can be used effectively in place

of anti-CD8 antibodies to cross-link surface CD8, for purposes of inducing immune non-responsiveness in the CD8-bearing cell. Different forms of multimeric α_3 polypeptides can be used in this context, for example, 5 chimeric polypeptides incorporating two or more α_3 polypeptide units, and separated by a polypeptide spacer, or conjugated polypeptides incorporating two or more α_3 polypeptide units covalently cross-linked by any one of a number of methods familiar to those in the art, including 10 a chemical agent, such as an aldehyde fixative, or a homobifunctional or heterobifunctional cross-linker can be used. Multimeric α_3 polypeptides can be administered to a patient alone, or in combination with other immunosuppressive agents, for example, anti-CD4 antibodies in 15 order to immunosuppress both CD4- and CD8-positive T-cell subsets simultaneously.

Cytodestruction of CD8-positive T-cells can also be achieved using α_3 polypeptides. In this instance, the α_3 polypeptides are used as targeting ligands to direct a 20 cytotoxic molecule selectively to CD8-positive T-cells. Any of a number of cytotoxic molecules can be covalently linked with an α_3 polypeptide for this purpose, for example, ricin, or Staphylococcal endotoxin, or a radionuclide.

25 α_3 :toxin conjugates can be used therapeutically in vivo in several ways. For example, CD8-positive tumor cells, such as CD8-positive lymphoma cells, can be eliminated in a patient suffering from such a tumor, by infusing α_3 :toxin conjugates into the patient. Clinical 30 protocols have been developed for the optimal use of polypeptide:toxin conjugates for therapy of cancers, such as for the use of anti-tumor antibody:toxin conjugates; methods for using α_3 :polypeptide conjugates for cancer therapy parallel these methods. In addition, α_3 35 polypeptide:toxin conjugates have application for non-cancer diseases. For example, α_3 polypeptide:toxin conjugates can be used to nonspecifically eliminate CD8-

positive T-cells to interfere with an acute disease flare in an autoimmune disease such as multiple sclerosis. This parallels current attempts to use anti-CD4 antibodies to eliminate CD4-positive T-cell function in the same clinical context. Other clinical applications for α_3 polypeptide:toxin conjugates in the treatment of alloimmune and certain infectious diseases, such as human immunodeficiency virus infection, will be apparent to those familiar with the art.

One particularly useful α_3 polypeptide is that incorporating the α_3 domain sequence exemplified above of HLA-A2.1 and maltose binding protein sequence. It will be obvious to those skilled in the art how to design alternative α_3 polypeptides that retain CD8 binding capacity. There is flexibility in the boundaries for the α_3 domain that can be chosen within HLA-A2.1 sequence, since all that is required for efficient CD8-binding function is sufficient sequence within the α_3 sequence to generate the β -pleated sheets and intervening sequences, together including the immunoglobulin-fold of the α_3 domain. Similarly, there is flexibility in the choice of second molecules to be appended to the α_3 domain within α_3 polypeptides, since the α_3 domain does not require extraneous sequences in order to assume its proper folded structure. The physical binding assay described in the previous example can be used as a general method, according to this invention, to evaluate the function of any chosen α_3 polypeptide in vitro. That is, those in the art can use the methods described herein, and well known in the art, to devise small or larger variants of the α_3 domain linked or not linked to a second molecule. Such a composition can then be tested to ensure binding to CD8 α or β . Those constructs which bind are useful in this invention.

Cytotoxic T-cell Immunosuppressive Therapy - In Vitro

In addition to serving as in vivo therapeutics, α_3 polypeptides can also be used as effective in vitro therapeutics. In the latter case, the α_3 polypeptides are also being used as reagents to suppress cytotoxic CD8-positive T-cell function. Purified GPI-modified proteins have the property of being reincorporable back into cell membranes. Any protein can be produced in a GPI-modified form by transfecting cells with an expression vector comprising a coding sequence for the protein of interest linked in-frame to the coding sequence for the 3'-end of a protein that is naturally GPI-modified and comprises the GPI modification signal sequence, for example, human decay-accelerating factor. Alternative 3'-end sequences from GPI-modified proteins can be employed in this way in order to situate the α_3 domain at variable distances from the cell membrane.

GPI chimeras can be readily purified by immunoaffinity, using anti- α_3 antibodies or soluble CD8, as disclosed in the present invention, conjugated to a solid phase matrix. Alternative methods for exogenous reincorporation into membranes can be employed. Standard methods include micellar transfer in the presence of low concentrations of detergent, for example, 0.004% NP-40. Reincorporation can also be efficiently accomplished, as disclosed in the present invention, by α_3 :GPI protein transfer in the absence of detergent. Detergent-free α_3 :GPI can be readily prepared by eluting the α_3 :GPI from the affinity column in the presence of CHAPS, a dialyzable detergent, and then dialyzing the CHAPS away from the α_3 :GPI. Detergent-free protein transfer can be used more generally for proteins other than α_3 polypeptides.

Alternative α_3 polypeptides and methods can be used for coating cells. For example, cells can be pre-coated with biotin:lipid conjugates or directly biotinylated. In turn, α_3 :streptavidin chimeras, as disclosed in the present invention, can be combined with the biotin-bearing cells

in order to coat them. High levels of surface α_3 can be obtained by this method.

α_3 polypeptides can also be expressed at cell surfaces by gene transfer. Any one of a number of expression
5 vector systems can be used for this purpose, for example, episomal vectors and retroviral vectors. Alternative membrane-anchoring domains can be incorporated into α_3 polypeptides. For example, those comprising a GPI-anchoring domain, a non-MHC transmembrane domain
10 comprising a hydrophobic polypeptide sequence, with or without an associated cytoplasmic extension, a MHC transmembrane domain, which can include the transmembrane domain naturally associated with the α_3 domain being used, or else, the transmembrane domain from a different class
15 I MHC molecule, with or without an associated cytoplasmic extension.

Once produced, a cell bearing an α_3 polypeptide can be used in a number of therapeutic contexts to modulate CD8-positive cells. Both in vitro and in vivo applications
20 will be readily apparent to those familiar with the art.

α_3 Polypeptide Production

The present invention discloses methods for producing α_3 polypeptides. Significantly, α_3 polypeptides can be produced using prokaryotic expression vectors. Hence,
25 unlike many other eukaryotic proteins, one is not restricted to eukaryotic expression vectors for production of this particular eukaryotic protein. The possibility of producing α_3 polypeptides by prokaryotic means makes these reagents economical.

30 Numerous prokaryotic and eukaryotic expression systems are generally available and can be readily applied to the production of α_3 polypeptides. Once α_3 -producing cells are generated by gene transfer, α_3 polypeptides can be purified from these cells by conventional protein
35 purification methods. In view of the capacity of α_3 polypeptides to bind CD8, soluble CD8 can be readily bound

to a solid phase matrix, and in turn, the CD8:solid phase matrix conjugate can be used for affinity purification of α_3 polypeptides.

In order to simplify α_3 polypeptide purification, polypeptide tags can be linked in-frame to α_3 domain sequences. For example, epitope tags can be appended to α_3 domain sequences to permit immunoaffinity purification with epitope-specific antibodies. An example of a commonly used epitope tag is a short sequence derived from a viral hemagglutinin. Other epitope tags are generally available or can be readily designed. Instead of an epitope tag, three or more histidines can be appended to either the amino or carboxy terminus of the α_3 polypeptide, in order to permit its rapid one-step purification by standard nickel-sepharose chromatography. Protease cleavage sites can be inserted in between either epitope tags or poly-histidine stretches and α_3 polypeptides to permit dissociation of the two following purification.

α_3 -specific Antibody Production

The present invention discloses an efficient method for producing α_3 -specific monoclonal and polyclonal antibodies. The findings described in the present invention disclose that at least one "pan-HLA" antibody is reactive with an α_3 domain epitope. However, in this case, the α_3 polypeptides are combined with the target T-cells in vitro in order to inactivate and/or eliminate the pathogenic T-cells from a cell population prior to infusion of the cell population into a patient.

Preferred in vitro therapeutic applications for α_3 polypeptides relate to hematopoietic stem cell transplantation, including, but not restricted to, bone marrow transplantation. For example, one significant therapeutic application is in autologous bone marrow transplantation to be performed in cancer patients who require bone marrow reconstitution after undergoing chemotherapeutic and/or radiotherapeutic insults to their

hematopoietic stem cell pool. In particular, lymphoma or leukemia patients to be so treated require purging of CD8-positive lymphoma or leukemia cells from their bone marrow prior to its autologous reinfusion. α_3 polypeptide:toxin
5 conjugates offer a preferred method for purging marrow of CD8-positive lymphoma cells. Methods for pre-treating marrow, for example, with antibody:toxin conjugates, have been well-described in the literature, and these methods can be readily adapted for use with α_3 polypeptide:toxin
10 conjugates.

Another significant therapeutic application is in allogeneic bone marrow transplantation to be performed in treating patients with a wide variety of disease conditions, including cancer. In this instance, the
15 therapeutic goal is to eliminate alloreactive CD8-positive T-cells from the donor marrow, in order to abrogate graft-versus-host cytotoxicity mediated by these cells. The cells can be inactivated using α_3 polypeptide multimers or destroyed using α_3 polypeptide:toxin conjugates.

20 CD8-positive T-cell Diagnostic Assays

The disclosure of α_3 polypeptides as effective CD8-binding reagents has diagnostic implications. α_3 polypeptides can be substituted for anti-CD8 antibodies or anti-CD8 antibody derivatives in a wide range of
25 diagnostic assays performed in vitro or in vivo. Advantages for α_3 polypeptides over antibodies have been described herein. From the standpoint of diagnostic assays which require large quantities of detecting reagent, a particular advantage for α_3 polypeptides is that
30 they can be produced in quantity using inexpensive prokaryotic expression systems.

A preferred diagnostic method utilizing α_3 polypeptides is to detect and quantify CD8-positive cells in mononuclear cell samples from patients. For example,
35 ratios of CD4:CD8 positive cells are determined in diagnosing and monitoring the clinical course of acquired

immunodeficiency disease. CD8-positive cells in the mononuclear cell populations can be detected by flow cytometry using α_3 polypeptide:fluorescein conjugates. Methods for obtaining mononuclear cell populations from the peripheral blood of patients, as well as methods for processing cells for flow cytometry, are well-established in the literature. It will be apparent that α_3 polypeptides can be tagged with a variety of other well-characterized reporters, such as biotin, streptavidin, peroxidase, and used in diagnostic assays. One preferred method involves chimeric polypeptides in which a tagged α_3 polypeptide reagent consists of a chimeric polypeptide, comprising an α_3 polypeptide linked in-frame, with or without a bridging polypeptide spacer, to streptavidin. Other polypeptide:streptavidin chimeras have been reported, and these have been shown to retain biotin-binding capacity. Hence, methods for producing and properly using α_3 polypeptide:streptavidin chimeras will be readily apparent to those skilled in the art.

Another preferred diagnostic method utilizing α_3 polypeptides is to detect and quantify CD8-positive lymphoma cells in vivo. α_3 polypeptides for this purpose can be tagged with any of a number of radionuclides that are suited for in vivo detection by standard radiological techniques. By this diagnostic method, solid lymphoma masses can be detected at various sites throughout the body of a patient, for example, bone marrow, spleen, liver, brain. At the present time, antibodies are generally used for such diagnostic tumor mass detection purposes. α_3 polypeptides have special advantages over antibodies for the detection of CD8-positive lymphoma masses, for example, the former are smaller and hence will penetrate tissues in a more optimal fashion.

There is no reported method for selectively generating α_3 -specific antibodies. Current immunization strategies that employ a polypeptide comprising the complete extracellular domain of a class I MHC molecule,

including the noncovalently associated β_2m light chain, as an immunogen do not efficiently yield α_3 -specific antibodies, since the α_1 and α_2 domains are substantially more immunogenic. Hence, the disclosure in the present invention that the α_3 domain can fold properly in the absence of other molecular structures normally associated with it provides a selective immunogen for generating anti- α_3 antibodies. Methods for generating monoclonal and polyclonal antibodies with recombinant polypeptides are well-known to those familiar with the art.

Coating Membranes with α_3 polypeptides

The present invention discloses that not only soluble, but also cell surface-associated α_3 polypeptides retain CD8-binding capacity. Hence, α_3 polypeptides can be incorporated into the surfaces of cells in order to modify their functional interactions with CD8-bearing cells. For example, α_3 polypeptides can be incorporated into antigen-presenting cell surfaces in order to modify their interactions with CD8-positive T-cells. Such incorporation will enhance adhesive interactions between the two cell types. Moreover, in certain circumstances, α_3 polypeptides can inhibit activation of the CD8-positive T-cells, functioning like multimeric α_3 polypeptides.

Different α_3 polypeptides are suitable for cell surface coating. For example, a preferred α_3 polypeptide for this purpose is a glycosyl-phosphatidylinositol (GPI)-modified form of α_3 .

Other embodiments are within the following claims.

Claims

1. Composition comprising a polypeptide comprising the α_3 domain, but lacking the α_1 and α_2 domains, of a class I major histocompatibility complex heavy chain polypeptide.
5
2. The composition of claim 1, wherein said α_3 polypeptide is soluble.
3. The composition of claim 1, wherein said α_3 polypeptide is membrane-binding.
- 10 4. The composition of claim 1, wherein said α_3 domain is linked to a non-MHC molecule.
5. The composition of claim 4, wherein said non-MHC molecule comprises an antigenic polypeptide.
- 15 6. The composition of claim 5, wherein said antigenic polypeptide comprises maltose binding protein.
7. The composition of claim 5, wherein said antigenic polypeptide comprises a portion of a viral hemagglutinin.
8. The composition of claim 4, wherein said non-MHC molecule comprises three or more histidines.
20
9. The composition of claim 4, wherein said non-MHC molecule comprises the Fc region of an immunoglobulin molecule.
10. The composition of claim 4, wherein said non-MHC molecule comprises a glycosylphosphatidylinositol moiety.
25

11. The composition of claim 4, wherein said non-MHC molecule comprises a toxin or a radionuclide.

12. The composition of claim 4, wherein said α_3 polypeptide comprises the transmembrane and/or cytoplasmic
5 domains of a non-MHC molecule.

13. The composition of claim 1, wherein said α_3 polypeptide comprises the transmembrane and/or cytoplasmic domains of a class I major histocompatibility complex molecule.

10 14. The composition of claim 1, wherein said α_3 polypeptide comprises more than one α_3 domain covalently linked together.

15. The composition of claim 1, wherein said α_3 polypeptide is not associated with β_2 -microglobulin.

15 16. The composition of claim 1, wherein said α_3 polypeptide is associated with β_2 -microglobulin.

17. An expression vector comprising a gene sequence encoding a polypeptide comprising the α_3 domain, but lacking the α_1 and α_2 domains, of a class I major
20 histocompatibility complex heavy chain.

18. The vector of claim 17, wherein said vector is prokaryotic.

19. The vector of claim 17, wherein said vector is eukaryotic.

25 20. A transfected cell comprising the vector of claim 17.

21. The cell of claim 20, wherein said cell is prokaryotic.

22. The cell of claim 20, wherein said cell is eukaryotic.

5 23. Method for reducing CD8-positive T-cell proliferation or cytotoxicity, comprising the step of contacting said CD8-positive T-cell with an α_3 polypeptide comprising the α_3 domain, but lacking the α_1 and α_2 domains, of a class I major histocompatibility complex heavy chain.

10 24. The method of claim 23, wherein said combining is performed in vivo.

25. The method of claim 23, wherein said combining is performed in vitro.

15 26. The method of claim 23 comprising eradicating a CD8-positive tumor cell from bone marrow to be transplanted, comprising the step of contacting said bone marrow with said α_3 polypeptide.

20 27. The method of claim 23, comprising eradicating a CD8-positive tumor cell in a patient with cancer, comprising the step of administering to said patient a therapeutic composition comprising an α_3 :toxin or α_3 :radionuclide polypeptide.

25 28. The method of claim 23, comprising eliminating alloreactive CD8-positive T-cells from bone marrow to be transplanted, comprising the step of contacting said bone marrow with said α_3 polypeptide.

29. A diagnostic method for detecting soluble CD8 or cell surface-associated CD8 in a patient samples, comprising the step of contacting an α_3 -containing

polypeptide comprising the α_3 domain of MHC-I and lacking the α_1 and α_2 domains, with said sample.

30. Method for purifying a polypeptide comprising an MHC-1 binding portion of CD8, comprising the steps of:

- 5 (a) covalently linking an α_3 -containing polypeptide comprising the α_3 domain and lacking the α_1 and α_2 domains of MHC-I to a solid phase support;
- (b) contacting a mixture comprising said CD8-containing polypeptide with the α_3 -containing
- 10 polypeptide:solid phase support conjugate; and
- (c) eluting the CD8-containing polypeptide from the solid phase support.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/05065

A. CLASSIFICATION OF SUBJECT MATTER IPC 5 C12N15/63 A61K37/02 A61K47/48 A61K49/02 C12N1/21 C12N5/10 G01N33/68 C07K3/18		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 5 C12N A61K G01N C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE JOURNAL OF IMMUNOLOGY vol. 150, no. 8(2) , 15 April 1993 , BALTIMORE MD, USA page 288A J. FAYEN ET AL. 'Soluble human CD8alpha binds to a recombinant fusion protein which incorporates the class I MHC alpha3 domain.' see abstract 1647 --- -/--	1-6, 15, 17-30
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : 'A' document defining the general state of the art which is not considered to be of particular relevance 'E' earlier document but published on or after the international filing date 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) 'O' document referring to an oral disclosure, use, exhibition or other means 'P' document published prior to the international filing date but later than the priority date claimed 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art 'Z' document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
1 September 1994		- 9. 09. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer Nooij, F

INTERNATIONAL SEARCH REPORT

Internati Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>TRANSPLANTATION PROCEEDINGS vol. 25, no. 1 , February 1993 , NEW YORK NY, USA pages 483 - 484 A. KRENSKY ET AL. 'Peptides corresponding to the CD8 binding region of HLA class I block the differentiation of cytotoxic T lymphocyte precursors.' see the whole document ----</p>	<p>1-3,13, 15,20, 22,23,25</p>
P,X	<p>WO,A,93 08817 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 13 May 1993 see claims; examples -----</p>	<p>1-30</p>

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/05065

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claim 23 (partially, as far as an in vivo method is concerned) and claims 24 and 27 (both completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

Information on patient family members

PCT/US 94/05065

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9308817	13-05-93	AU-A- 3062892	07-06-93
		CA-A- 2122266	13-05-93

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